INTERFERON INDUCERS AGAINST INFECTIOUS DISEASES

ANNUAL REPORT

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A series of interferon inducers was developed, in which carboxymethyl cellulose (the undesired component of poly ICLC) was replaced by other anionic polymers or by graft polymers of poly(L-lysine) with saccharides. Most of these showed effective antiviral action against Rift Valley Fever virus and Punta Toro virus. Most were less toxic than poly ICLC and were non-immunogenic. The promising replacements for carboxymethylcellulose are: carboxymethylamylose, carboxymethyl-β-cyclodextrin, gelatin, sulfated gelatin, sulfated β-cyclodextrin, poly(lysine)-dextran graft, and poly(lysine)-glucose graft.

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Foreword.

Citations of commercial organizations and trac names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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1. Problem Under Study.

The double-stranded synthetic polynucleotide poly I.poly C (i.e., poly-inosinic acid-poly-cytidylic acid, also to be called poly IC or IC) is an interferon (IFN) inducer. In primates it is not effective as an antiviral agent, presumably because of circulating nucleases which quickly degrade it. When complexed with cationic poly L-lysine (PLL) and anionic carboxymethylcellulose, CMC, it is effective in humans (1). But there are reservations about CMC. It appears not to be excreted or metabolized, and it is suspected as a carcinogen (2).

The problem being addressed in this research is the preparation of effective, safe IFN inducers devoid of CMC. In addition to seeking formulations which are effective IFN inducers, we are also seeking formulations which are effective antiviral agents. The formulations should be less toxic than ICLC, non-immunogenic, and metabolizable or excretable. To these ends our main efforts have been to develop anionic polymers to replace CMC. We are also seeking to replace both PLL and CMC by modifying the PLL with engrafted polysaccharides.

2. Background

A number of candidates have been developed in this work, which show a range of IFN-inducing ability, antiviral action, and reduced toxicity (compared with poly ICLC), which make them promising candidates for clinical testing. Future research will be focused on the selection of the best one or two for clinical testing.

3. Rationale of the Research.

Since CMC has undesirable features, including non-excretion, non-metabolization, and possible carcinogenicity, we are seeking formulations without CMC. We are exploring two approaches to this goal. One is the replacement of CMC with other anionic biopolymers, selected on the basis of known or expected safety, and being excretable or metabolizable. Most of the CMC replacements were selected on the basis of a history of safe use as blood volume expanders, or being closely related to such. These include gelatin, anionically-modified gelatin, carboxymethyl polysaccharides, sulfated polysaccharides, and anionic cyclodextrin. The second approach is the use of poly L-lysine (PLL), covalently grafted to a saccharide (without anionic groups). The PLL portion would bind to the poly IC, through its cationic groups, while the grafted saccharide would provide a solubilizing and hydrating effect. PLL-dextran and PLL-sugar grafts are being studied. Another reason for studying these graft polymers is that dextran is readily cleared from the circulation of dogs (3). We graft saccharide to only a fraction of the PLL residues. It may be expected that a graft polymer will be cleaved by trypsin-like enzymes and the fragments produced will be dextran chains bearing terminal oligolysine.

A. Chemical Studies: CMC Replacements.

i. Anionic Polysaccharides as CMC Replacements, Carboxymethylated and Sulfated.

Carboxymethyl polysaccharides are anionic polymers in which the ionized carboxymethyl group has been introduced:

$$R\text{-}OH \quad \text{-->} R\text{-}O\text{-}CH_2\text{-}COO^- \qquad R = \text{Saccharide}$$

The carboxymethyl polysaccharides investigated as CMC replacements are the following:

a. CMdextran (CMD). During the first two years we examined CMdextran of 10 kDa and 40 kDa molecular weight, and with degrees of substitution (DS) of about 0.2 to 2 per average glucose residue. The work of Chang et al. (3) showed that CMdextran is about 50-60% cleared from the circulation in dogs in about 1/2 hour, with no difference as to molecular weight of the CMdextran. The formulations of poly IC, PLL and CMD were effective IFN inducers in mice.

b. CMamylose (CMA). This was selected in expectation of low toxicity, and because of a report from China (4) (on its use as a blood volume expander) that it is non-immunogenic and is rapidly cleared from the body. In the work done so far, molecular weight has not been a concern. If results warrant it, we shall study the use of different molecular weights.

c. CM-β-cyclodextrin. β-cyclodextrin is a small, cyclic glucose heptamer. It was carboxymethylated to introduce negative charges. It was thought that β-cyclodextrin would have several advantages, which are described in detail below, related to excretion, purity and reproducibility inherent in a small molecule of definite size, rather than a large polymer of a range of molecular weights.

Sulfated poly- and oligosaccharides were also selected, because sulfate esters will bind differently, may be of low toxicity, and more metabolizable than carboxymethyl derivatives. The sulfated saccharides are:

a. Amylose sulfate.
b. Amylopectin sulfate.
c. β-Cyclodextrin sulfate (for the same rationale as for CM-β-cyclodextrin, as well as the potential advantages of sulfate esters).

ii. Anionically Modified Gelatin

During the second and third years of the contract, we investigated inducer formulations containing sulfated gelatin and unmodified gelatin. Gelatin is metabolizable and has been used

as a plasma expander. Sulfated gelatin is prepared by reacting gelatin with sulfuric acid or chlorosulfonic acid. The numerous hydroxyl groups of gelatin (about 15 moles per 100 residues, or 9300 grams) react as follows:

$$R\text{-}OH \longrightarrow R\text{-}OSO_3H \xrightarrow{\text{neutralize}} R\text{-}OSO_3^-$$

The reactions are carried out with gelatin in cold sulfuric acid, or with gelatin dissolved in cold trifluoroacetic acid-sulfuric acid, or with chlorosulfonic acid on gelatin in H_2SO_4.

4. Experimental

I. Chemical Studies.

A. Preparation of Inducer Formulations.

a. General Procedure

The procedure of Levy et al. (1) was used to prepare poly ICLC and also formulations in which an polyanionic replacement for CMC was used. This involves preparing a PLL-polyanion complex, then mixing this with poly IC. For IC-(PLL-dextran), and the analogous IC-(PLL-glucose) and IC-(PLL-ribose) the PLL-saccharide graft is mixed directly with poly IC. Some experimental formulations required modification in the ratios of components or the final concentration.

b. Sterilization of Components.

Poly IC was obtained sterile from the vendor (Pharmacia). Solutions of PLL·HBr (Sigma), modified PLL's and modified gelatins were filtered through a 0.2 μ filter. The sterile components were mixed under a sterile hood and sealed in sterile serum vials.

It was found that standard butyl rubber septa emitted colored and turbid impurities into sterile saline. To eliminate this source of impurity, we boil the septa in water, which removes the light absorbing and turbid materials, or we use silicone septa which do not liberate impurities (at any rate impurities detectable by spectrophotometry.)

c. Quality of Saline Solutions.

Our inducer formulations have been made in sterile saline, using saline solutions packed for human use, injection or irrigation. These are packed in plastic. We examined sterile saline by light scattering and found that some lots gave markedly increased scattering at elevated temperature, increasing linearly up to 3-fold between room temperature and 90° C. Saline prepared in glass by us did not show this effect. The scattering effect

of commercial saline probably arises from the plastic container, either from the polymer or from a plasticizer. The increased scattering at elevated temperature is suggestive of hydrophobic aggregation. As a result we routinely use saline made in glass.

B. Carboxymethylation of Polysaccharides.

The carboxymethylation is done by the procedure of Chang et al. (3), with modification. A typical experiment is carried out as follows: To a solution of 4 g of polysaccharide in 35 mL of NaOH solution (15 g of NaOH in 100 mL of water) at 60° C, is added 5.8 g of chloroacetic acid over 4 minutes. After 90 minutes the pH is brought to about 4.8 with glacial acetic acid, and the solution is dialyzed against water and freeze-dried. The dialysis procedure is used for CMamylose, but carboxymethyl dextran is precipitated with methanol, dissolved in water and reprecipitated.

C. Preparation of Carboxymethyl-β-Cyclodextrin.

This reaction was carried out by a modification of the foregoing carboxymethylation procedure. To 2.2 g of β-cyclodextrin in 5 mL water was added 2.34 g of sodium chloroacetate. NaOH (10 M) was added in small portions over 2 hr., until 2 mL had been added. After standing overnight at room temperature, the solution was heated at 50° for 4 hours. Methanol, 20 mL, was added to precipitate the CM-β-cyclodextrin, followed by filtration and washing with 30 mL methanol, and vacuum-drying. The product was dissolved in 2 mL H_2O and passed through a column of Sephadex G-10, to separate it from NaCl and sodium glycolate (byproducts of the reaction). The fractions were tested for CM-cyclodextrin and cyclodextrin with iodine. These gave brown and purplish spots, respectively, on paper with iodine. NaCl-containing fractions were detected with $AgNO_3$.

D. Titration of carboxymethyl groups.

The sample is dried in vacuo over silica gel. A portion of about 50 mg is dissolved in 50 mL of water. For the titration of starting polysaccharide 100-150 mg is dissolved in 50 mL water. A volume of 15 mL is made 0.1 M in KCl with 2 M KCl, and titrated with 0.1 M HCl to about pH 2.25. A blank of the same volume of 0.1 M KCl is titrated, and a difference titration curve is drawn. Below pH 2.4 the difference curve is constant on the volume axis, indicating that the titration is complete.

E. Quality of Poly(L-lysine) Lots.

The poly(L-lysine), PLL, used must meet several criteria for suitability: absence of residual carbobenzyloxy (CBZ) protecting groups, absence of excessive light scattering, adequate molecular weight and, in general, having the characteristic conformational properties of PLL. Every batch of PLL is tested by spectrophotometry and circular dichroism. The substantial

absence of residual carbobenzyloxy groups is demonstrated from the absorption spectrum in the benzene range, 280-250 nm, using a concentration of 5 or 10 mg/mL, which permits detection of one CBZ group per 1000 lysine residues. CBZ below about 2 per 1000 is considered satisfactory. The same spectrum also gives information on the presence of light scattering, which would be manifested as a rising slope at 350-300 nm. Light scattering would be evidence of aggregation or cross-linking. The spectrum also gives notice of the presence of other absorbing impurities.

F. Synthesis of PLL-Dextran and PLL-Sugar Grafts.

To 0.06 g of PLL-HBr in 5 mL H_2O was added 1 g of dextran and 0.063 g (1 mmole) $NaCNBH_3$, at room temperature. After two weeks of stirring, the reaction mixture was dialyzed against water and freeze-dried to yield 0.69 g of product. The crude material is purified on a 0.7 X 40 cm column of AG-50-X2 (protonated form). Unreacted dextran is eluted first with 200 mL H_2O, and the PLL-dextran graft is eluted with 400 mL 0.5 HCl, dialyzed against water and freeze-dried (yield 0.2 g). Then the product is put through Sephadex G-100, eluting with 0.2 M NaCl. Four peaks are obtained, representing unreacted PLL and three products. The latter three are not well resolved, as expected for a series of species of varying degrees of substitution.

PLL-ribose and PLL-glucose grafts were prepared similarly using one-tenth as much sugar as dextran, but were not chromatographed.

G. Melting Profiles.

Inducer complexes were diluted to contain 50 µg/mL of IC. Absorption spectra were recorded at room temperature from 325 nm to about 240 nm, using cells of 1 cm optical path and a Cary 219 or Uvikon 860 spectrophotometer, to ascertain that the spectra were normal and to reveal any significant degree of turbidity (manifested as absorbance at long wave length. Melting profiles were recorded at 246 nm with the Cary 219 Tm accessory, or (for the Uvikon spectrophotometer, a platinum resistance thermometer (PRT) and X-Y recorder operated off the spectrophotometer output and the PRT output), and a Neslab circulating bath driven by a Neslab temperature programmer at 1° per minute.

H. Preparation of Sulfated Gelatin.

A gelatin solution (1%) is freeze-dried. The dry fibers are sulfated by one of the following procedures.

1) Gelatin (0.5 g) is dissolved in 9 ml of ice-cold concentrated H_2SO_4, stirred occasionally during 1 hour, and poured into 100 mL of ice-cold 4.7 M sodium acetate, pH 7. The gummy lump of precipitated SO_4gelatin is dissolved in H_2O (with gentle warming if needed) and dialyzed exhaustively against H_2O, and freeze-dried.

2) Gelatin (0.5 g) is dissolved in 10 mL ice-cold trifluoroacetic acid, 10 mL cold H_2SO_4 is added. The rest of the procedure is as above, except that 130 mL of 4.7 M sodium acetate is used.

Freeze-dried material is dissolved in water and treated with mixed-bed ion-exchange resin (H^+ and OH^-). A pH of about 2.8 or less is indicative of a major degree of substitution.

I. Synthesis of Sulfated Saccharides.

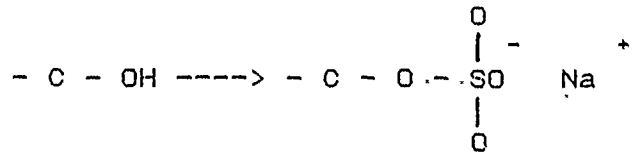
β-cyclodextrin and amylose were sulfated with $SO_3 \cdot (CH_3)_3N$ in dimethylformamide, at temperatures and reactant ratios adjusted to obtain the desired degrees of sulfation.

For β-CD, the reaction was carried out at 60-65° for 36 hrs, solvent was removed under vacuum, the syrup was extracted with methanol, and the methanol was cooled to 2°, resulting in the precipitation of the product. Product was purified by dissolving in water with NaOH (to pH 10), and the β-$CDSO_4$ was precipitated with methanol, washed with methanol and tetrahydrofuran.

For amylose-SO_4 the reaction mixture was heated at 60-65° for 24 hr, then cooled to 5°, and the solvent was decanted. The residue was dissolved in H_2O with NaOH (pH 10), filtered and dialyzed against 2% $NaHCO_3$, then against H_2O, and freeze-dried. The degree of sulfation is proportional to the ratio of $SO_3 \cdot N(CH_3)_3$ to amylose. Amylopectin was sulfated similarly.

The degree of sulfation was determined from elemental analysis for sulfur. A secondary method was developed: the melting/decomposition temperature of sulfated amylose was found to be a monotonic function of the degree of sulfation.

The sulfation reaction results in the conversion of hydroxyl groups to anionic sulfate esters (sodium salts):



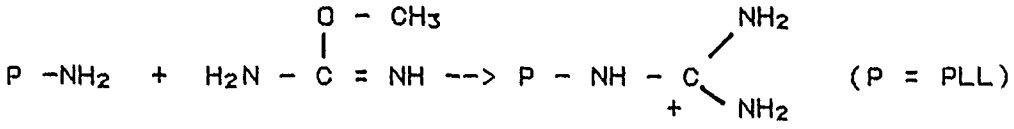
J. Methylated and Guanidinated PLL Derivatives.

a. Methylated PLL and methylated PLL-dextran grafts. Methylation was carried out on the polymer in H_2O at pH 9-10, room temperature, with dimethyl sulfate (10-fold excess), followed by neutralization, dialysis against 0.1 M NaCl, and then H_2O, and freeze-drying. Methylation was shown to be complete by ^1H nmr. The reaction is:

$$-NH_2 \; + \; (CH_3\text{-}O)_2SO_2 \; \longrightarrow \; -N(CH_3)_3^+$$

b. Guanidinated PLL and PLL-dextran grafts. Guanidination was carried out on the polymer in H_2O at pH 9.5 with O-methyl-iso-urea·SO_4 (10-fold excess) followed by dialysis against 0.1 M NaCl, then against H_2O and freeze-drying. ^{13}C nmr showed the presence of guanidine groups.

The reaction is:



$$P\text{-}NH_2 + H_2N\text{-}C(=O\text{-}CH_3)\text{=}NH \longrightarrow P\text{-}NH\text{-}C^{+}(NH_2)(NH_2) \qquad (P = PLL)$$

II. Biological Studies

A. Interferon Induction in Mice.

The interferon inducers were evaluated for interferon production in BALB/c mice. Each inducer was compared to a standard poly ICLC preparation. Twenty gram mice were given a single i.v. injection of inducer containing 10 g of poly IC. Blood was obtained by orbital bleeding 3 hours after injection. Usually there were 8 treated mice per group plus 2 mice that received placebo. Serum was assayed for interferon.

B. Interferon Assays.

Three-fold dilutions of serum were made in Minimal Essential Medium (MEM) containing 5% fetal bovine serum (FBS). The dilutions were done in 96-well microtiter plates, followed by addition of 20,000 murine L-cells/well. The cultures were incubated for 18-20 hours, the trays inverted to remove the medium, and vesicular stomatitis virus, at a multiplicity of infection of 0.15 plaque-forming units per cell, in MEM containing 2% FCS, was added to the cells. The cultures were incubated until virus controls showed marked cytopathic effects (24-48 hours). The medium was removed and antiviral activity determined by a standard colorimetric procedure which measures uptake of a vital dye, neutral red. Interferon titers are expressed in international reference units based upon standards received from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, NIH.

C. Antiviral Effect.

Formulations were submitted to Dr. Meir Kende, Fort Detrick, for testing against Rift Valley Fever virus in mice, and to Dr. R. Sidwell, Utah State University for testing against Punta Toro virus in mice.

D. Toxicity Testing (LD_{50}).

LD_{50} was measured by injecting mice with doses of formulation containing 100, 200, 400, 600, 800 and sometimes 1000 g of IC and observing the number surviving after 12 days.

E. Safety testing.

Selected inducers were evaluated for effect on weight in mice and guinea pigs according to the FDA Code of Federal Regulations on general safety.

Four Balb/c mice (16-20 g) and two Hartley guinea pigs (300-400 g) were injected intraperitoneally (i.p.) with 8 mg/kg of inducer in 0.5 ml or 5.0 ml, respectively. This dose was determined by results of experiments in which various doses of the standard inducer, Poly ICLC, were injected into mice and guinea pigs.

Results with the selected inducer are compared to those obtained with the Poly ICLC standard.

F. Testing for Immunogenicity.

a. Immunization protocol.

Ten groups of 6-8 week old female BALB/c mice, 5-6 per group, were used. The mice in each group were injected subcutaneously with one of the test antigens. The first injection consisted of 100 µg of the material in 0.1 or 0.2 mL of a 1:1 emulsion of Freund's complete adjuvant and the PBS solution of the material. Injections were with 1 mL disposable syringes, and although the emulsion is very viscous 25-guage needles were successfully used. After 3-week and 6-week periods a similar injection, but using Freund's incomplete adjuvant, was given.

b. Collection of serum.

About two weeks later the mice were bled by cutting off about 1/2 inch of the tail and letting the blood drip into a 1.5 ml plastic centrifuge tube. This method of bleeding consistently produces a better yield, is faster and easier than bleeding from the heart. Furthermore, the mice survive and can be again bled after a few days by removing 1/8-1/4" more of the tail; this can be done a number of times.

The tail-bleeding is facilitated and only really successful if the mice are warmed-up under an infra-red light for 4-5 minutes before bleeding. The whole group of mice is initially warmed-up and kept warm as individual mice are bled. Warming should be to a substantial degree and can be monitored by holding one's hand near the mice and by observing the activity of the mice. It is well to place a paper towel on the cage bottom (no sawdust) since the bare cage bottom may get uncomfortably hot.

An individual mouse is removed from the cage by the tail and grasped firmly and gently in a crumpled paper towel with the tail held projecting from the towel by the thumb and forefinger. The tip of the tail (on a firm surface) is cut off with a razor blade and the drops of blood then permitted to fall into the centrifuge tube. With experience, about 15 good sized drops can be collected in less than a minute. The blood flow can be stopped by squeezing the tail for a half minute or so. A little bleeding may follow briefly when the mouse is returned to a clean cage but all of the mice have always survived.

Following overnight clotting of the blood, the tubes are centrifuged (Microfuge) for a good recovery of clear serum. As much of the serum as can be withdrawn (along with some red blood cells) using a disposable pipet with a control, not a rubber bulb, is transferred to a second 1 1/2 ml centrifuge tube and this is centrifuged. From this tube a good yield of clear, cell-free serum can be withdrawn from the very small red cell pellet, and stored in the freezer.

c. Testing sera for antibodies.

To determine whether or not the sera contained antibody, a precipitation-in-gel procedure was used. In this procedure, a commercially available apparatus (LKB) permits one to pour an approximately 1.3 mm thick layer of molten 1.5% agar solution in buffer onto labelled 1 inch X 3 inch microscope slides, after which a hexagonal array of 3 mm holes, 8 mm between holes, and around a single central hole, is cut in the agar on each half of the slide.

The 5-6 sera obtained from each of the ten groups of mice were tested against one or more of the injected materials. Thus. 8-10 µL of each serum from the five individual mice injected with an antigen (say, carboxymethyl dextran) was placed in five individual wells of each of four of the hexagonal arrays on two labelled slides; the center well of each of the four arrays was then filled (8-10 µL) with one of the four solutions which the serum is to be tested against. Diffusion was allowed to proceed in a closed, moist chamber for 24 hours at room temperature; under these conditions, even the finest precipitin arcs ordinarily appear and little or no increase in the density of the arcs occurs with longer periods of time. Careful examination of the slides after 24, 48 and 72 hours revealed no precipitin arcs in any experimental case, but did show precipitin arcs (as expected) in the case of bovine serum albumin. Repetition of the procedure using an agar solution containing 0.5% polyethylene glycol which enhances the precipitin reaction in gel still did not result in any precipitin arcs.

5. Results.

A. Introductory Notes to the Results.

Each type of inducer candidate has three polymeric components, which means that many variations in composition are possible. In addition to the proportions of the components, other variables are molecular weight and degree of modification. In addition, reproducibility must be determined, using a biological assay subject to variability in mouse response. The exploration of the possible combinations is limited by our biological testing capacity.

i. IFN Induction vs. Antiviral Action.

We test our experimental agents for their ability to induce IFN in mice. IFN induction is measured by the ability of mouse blood serum to protect cells in culture against vesicular stomatitis virus. Direct protection of mice against a virus is not measured here. Since there is no clear understanding yet of the optimum IFN blood titers, we do not reject agents which induce modest IFN titers. Also, blood is taken three hours after injection of the inducer, which may not be near the time of peak IFN titer. As will be seen, modest IFN titers can accompany effective antiviral action. The latter is measured at Fort Detrick by Dr. Meir Kende, using Rift Valley Fever virus in mice and by Dr. R. Sidwell, at Utah State University using Punta Toro virus. Dr. D. Gan-Gemi has measured the time course of IFN induction of one inducer in a monkey species.

ii. Tables of IFN Induction.

All inducer tests are done with a single dose containing 10 g of IC per mouse. Each IFN titer for an experimental formulation shown is the average of the titers of 7-8 mice, and is compared with the average titer for 7-8 mice obtained with ICLC as the standard.

In the tables of IFN induction the composition of some inducers is given in actual mg/mL, for each component (in the order shown). The solution to be tested is diluted 1:40 with saline and a 200 µL dose is injected i.v. In the course of the work we discovered that standard septa for serum bottles and standard sterile saline contain avoidable impurities (see Experimental. We make our own sterile saline and use silicone septa.

iii. Digestion by Ribonuclease.

It has been standard procedure, beginning with the pioneering work of Levy on ICLC, to measure the rate of digestion of poly C in the ICLC complex. A low rate of digestion (relative to the digestion of simple poly IC) has been taken as indicating that the poly IC is complexed. This is not necessarily the case.

While a fast rate of digestion is usually indicative of uncomplexed poly IC, a slow rate is not necessarily indicative of the converse, because we have observed that some of the polyanions (i.e., CMC, CMamylose, amylose sulfate) are inhibitors of ribonuclease. Therefore, when a complex containing an RNase inhibitor is only slowly hydrolyzed by RNase, there is no way to tell if this is because of protection of poly IC within the complex or because of inhibition of the RNase. Therefore, we no longer routinely measure digestion by RNase, but do so only for formulations not containing an RNase inhibitor.

B. Summary of Results on Interferon Inducer-Antiviral Agents.

Results are presented below for IFN inducers based on poly IC, poly(L-lysine) and the replacement for carboxymethylcellulose (CMC). The results include IFN titers, antiviral action, LD_{50}, safety testing, and some physical data. The results are organized by the types of polymer being investigated as CMC replacements.

a. Poly(L-Lysine)-Dextran Grafts (IC-(PLL-dextran)).

Much of the work of the year was devoted to IFN inducers formulated from poly IC with graft polymers of poly(L-lysine), and dextran. Work of the previous year had shown these formulations to be promising. Although IFN induction in mice (at our standard blood sampling time of 3 hours after injection) was typically about 10-30% of that induced by poly ICLC (Table 1), there was good antiviral action and lower toxicity than for poly ICLC. During this year we have worked on verifying earlier data, improving the synthetic procedures, and exploring the effects of molecular weights and proportions of PLL and dextran combined into the graft polymers. Also, an initial pharmacokinetic study in a monkey showed that a formulation of IC-(PLL-dextran) induced as high a titer of IFN as did poly ICLC, but with a different time-course.

The dextran chains are attached to the PLL backbone through reaction of the terminal aldehyde of dextran with the amino groups of PLL. Complexes of the graft with poly IC have no anionic polymer (except, of course, the poly IC). Only a fraction of the amino groups of PLL are engrafted with dextran. In principle this should permit digestion of the PLL backbone to liberate fragments small enough to be eliminated.

Formulations with several ratios of PLL-dextran to poly IC were examined in the previous year, in order to find the best ratio. A ratio of 3.75 to 1, by weight, was adopted as standard.

i. Molecular Weights.

We next examined the effects of varying the molecular weights of the PLL and dextran. We used PLL of molecular weights 6000, 21,500, 38,000, 62,500 and 406,000, and dextrans of molecular weights of 10,000, 40,000 and 70,000, in various combinations. IFN inductions for these preparations are given in Table 1.

ii. Dextran-to-Lysine Ratio.

Most of the grafts were made with a constant input ratio of dextran to PLL, regardless of the molecular weights of the polymers. Experiments were also done with higher and lower ratios of dextran to PLL, namely twice and one-half the standard ratio (Table 1). These are lots IV-131 and IV-258 (2X) and lots IV-132 and IV-259 (1/2X). IV-132 induced more than three times as much IFN as did IV-131; similar differences have been seen among standard formulations. Because IV-132 had all of its poly IC complexed, it appears that the complexed poly IC is the more active component than is free poly IC. The range of IFN induction for these four are similar to the general variation in IFN induction.

LD_{50} for IV-132 is lower than for IV-131 (Table 2), but if it is a threefold better inducer, it may be more useful. There is no apparent correlation between IFN induction and molecular weight of the components, or the ratio of dextran to PLL.

Table 1. IFN Induction in Mice by IC-(PLL-dextran)[a]

	Mol. wt. of PLL (kDa)	Mol. wt. of Dextran (kDa)	IFN titer	IFN titer of poly ICLC	IFN titer as % of poly ICLC titer
IV-18	6	10	86	799	11
IV-19	6	10	35	799	4
IV-20	6	70	84	799	11
III-135A[b]	21.5	10	45	357	8
III-190[b]	21.5	10	151	710	21
III-230[b]	21.5	10	173	1235	14
III-232[b]	21.5	10	77	1235	6
III-285	21.5	10	242	1667	15
III-297	21.5	10	76	810	9
III-298	21.5	10	118	810	15
III-252	21.5	40	270	539	50
III-283	21.5	40	197	1667	12
III-253	21.5	70	204	539	38
III-284	21.5	70	253	1667	15
III-135B[b]	38	10	78	357	13
III-286	62.5	10	351	1667	21
IV-131[c]	62.5	40	133	1097	12
IV-132[d]	62.5	40	430	1097	39
IV-224	62.5	40	157	866	18
IV-258[c]	62.5	40	134	1128	12
IV-259[d]	62.5	40	220	1128	20
IV-225	62.5	70	127	1039	12
IV-51	406	10	307	1183	26
IV-52	406	70	264	1183	22

a. The composition of the inducer is, in mg/mL: poly IC, 2; PLL-dextran, 7.5. A dose of 10 µg (as contained poly IC) is injected. This is the standard dosage used in all of our induction experiments.

b. From Annual Report, April 1, 1989. Table 5.

c. PLL-dextran graft made with 2X the standard dextran to poly IC ratio.

d. PLL-dextran graft made with 1/2 the standard dextran to poly IC ratio.

Table 2. LD_{50}[a] of IC-(PLL-Dextran).

Compound Lot #	Molecular Weight of PLL, in kDa	Molecular Weight of Dextran, kDa	LD_{50}, mg/kg[a]
Poly ICLC[b]	-	-	11[c]
IC-(PLL-dextran)			
IV-18	6	10	32
IV-19	6	10	29
IV-20	6	70	27
III-285	21.5	10	>40
III-190	21.5	10	25
III-230	21.5	10	35[d]
III-232	21.5	10	30
III-297	21.5	10	43
III-298	62.5	10	48
III-286	62.5	10	>40
IV-131	62.5	40	42
IV-132	62.5	40	31
			average >35

a. Dose required to kill 50% of mice.
b. Standard ICLC.
c. Average of 4 tests (13, 11, 10, 9 mg/kg).
d. Average of 2 tests (33 and 37 mg/kg).

Although the IFN titers induced by most lots of IC-(PLL-dextran) are in the range of 10-30% of that of poly ICLC, they show effective antiviral activity. Our IFN titers are measured with blood drawn 3 h. after injection of the drug into mice. Pharmacokinetic studies would be needed to determine the times at which peak levels of each inducer occur. A start has been made. Dr. D. Gan-Gemi (School of Medicine, Univ. of South Carolina) has measured IFN induction by an IC-(PLL-dextran), lot III-252 in the cynomolgous monkey, with the following results (Table 3).

Table 3. Time Course of IFN Induction by IC-(PLL-Dextran) in Cynomologous Monkey.

	IFN Titer		
	4 h.	12 h.	24 h.
Poly ICLC	22	98	870
IC-(PLL-dextran)	0	800	340

(Lot III-252 gave an IFN titer of 270 units in mice at 3 h., 50% of that of a lower-than-average poly ICLC IFN titer).

In this primate IC-(PLL-dextran) peaked earlier than did poly ICLC, reaching essentially the same titer as did the latter, and still having at 24 h. 43% of its peak titer. This result shows that IC-(PLL-dextran) in one primate induces as high a titer as poly ICLC and does so earlier. This result emphasizes the need to obtain kinetic data in primates.

iii. Toxicity of IC-(PLL-Dextran).

The LD_{50} values for a number of lots of IC-(PLL-dextran) are given in Table 2. All lots of IC-(PLL-dextran), regardless of the molecular weights or proportions of components in the graft polymer, were less toxic than was poly ICLC. While sufficient data are not yet available for reliable statistics, there appears to be a trend toward the lowest molecular weight PLL (6 kDa) giving the lowest LD_{50} values. These materials also gave low IFN titers. Therefore, 6 kDa PLL will not be investigated further.

iv. Melting Profiles.

Melting profiles were run on the formulations of IC-(PLL-dextran) (Table 4). Four showed two melting transitions at 63-65° and 80-85° (III-252, 253, 283, 284). Two showed only one transition at 78° (III-285) or 83° (III-286). There was no correlation between single-step and two-step melting on the one hand and IFN induction on the other. For each of the two-step profiles, ΔA of the second constituted 60-70% of the total ΔA.

Table 4. Melting Transitions of IC-(PLL-dextran).

Lot	Molecular Weight PLL (kDa)	Dextran (kDa)	Tm_1	Tm_2
IV-18	6	10	-	73°
IV-19	6	40	-	75°
IV-20	6	70	-	73°
III-135A	21	10	-	85°
III-190	21.5	10	-	87°
III-230	21.5	10	66°	76°
III-232	21.5	10	-	83°
III-285	21.5	10	-	78°
III-297	21.5	10	-	80°
III-298	62.5	10	-	85°
III-252	21.5	40	64°	83°
III-283	21.5	40	63°	82°
III-253	21.5	70	64°	83°
III-284	21.5	70	63°	82°
III-135B	38	10	-	85°
III-286	62.5	10	-	83°
IV-224	62.5	40	67°	90°
IV-225	62.5	70	67°	87°
IV-131[a]	62.5	40	67°	87°
IV-258	62.5	40	68°	85°
IV-259	62.5	40	-	87°
IV-132[b]	62.5	40	-	88°
IV-51	406	10	-	85°
IV-52	406	70	73°	90°

[a] PLL-dextran, with 2X the standard dextran-to-lysine ratio.
[b] PLL-dextran, with 1/2 the standard dextran-to-lysine ratio.

v. Antiviral Action.

Thirteen lots of IC-(PLL-dextran) were tested against Rift Valley Fever virus (Table 5). The overall results were comparable to, or somewhat better than those with poly ICLC. The data show no correlation between anti-RVFV activity and molecular weights of the PLL and dextran components. There are yet to be tested lots made with twice and one-half ratios of dextran to PLL. Combined with the lower toxicity of IC-(PLL-dextran) these antiviral results show this class of formulation to be promising.

Table 5. Protection of Mice against Rift Valley Fever Virus by IC-(PLL-dextran).[a]

Preparation	Inducer dose µg/mouse	Survivors Experimental Day 22	Survivors Poly ICLC Standard Day 22
none	-	0	-
III-230	10	100	90
	2.5	100	100
III-232	10	90	90
	2.5	90	100
III-252	10	90	90
	2.5	50	100
III-253	10	100	90
	2.5	90	100
III-135A	2.5	100	87
III-135	2.5	80	100
III-190	2.5	100	70
IC(PLL-Dextran) III-283	10	100	80
"	2.5	100	50
IC(PLL-Dextran) III-284	10	90	80
"	2.5	100	50
IC(PLL-Dextran) III-285	10	100	80
"	2.5	100	50
IC(PLL-Dextran) III-286	10	100	80
"	2.5	100	50
IC(PLL-Dextran) III-297	10	100	80
"	2.5	100	50
IC(PLL-Dextran) III-298	10	100	80
"	2.5	100 (average 93%)	50 (average 78%)

[a] 10 mice per group except for lot III-135A (15 mice). 250 PFU of virus per mouse.

Lot III-190 of IC(PLL-Dextran) was tested against Punta Toro virus in mice (by Dr. R. Sidwell, Utah State University), with the results shown in Table 6. The results were compared with two tests of poly ICLC, and show lot III-190 to be as good as the average of the two tests on poly ICLC, except perhaps at the two lowest doses.

Table 6. Anti-Punta Toro Action of IC-(PLL-dextran).

Inducer dose mg/kg	Survivors			
	Lot III-232	Lot III-190	poly ICLC[a] I	II
1.0	9/10	10/10	-	-
0.5	9/10	10/10	10/10	-
0.25	9/10	10/10	-	-
0.125	9/10	10/10	10/10	9/10
0.1	6/10	10/10	10/10	9/10
0.032	1/10	7/10	9/10	3/10
0.01	1/10	1/10	9/10	3/10
0.0032	0/10	0/10	3/9	1/10

[a] Blanks, not determined.

The results for IC-(PLL-dextran) show IFN induction and antiviral action comparable to that of poly ICLC, combined with lower toxicity.

b. PLL-Monosaccharide Grafts.

The PLL-dextran grafts described above contain long polysaccharide chains. We decided to examine the possibility of using short chains, beginning with the monosaccharides ribose and glucose. We considered it possible that the use of small saccharide might facilitate metabolism or excretion. Another advantage is that small sugars are of defined molecular weight, thereby one avoids the complications arising from the molecular weight distribution of polymeric saccharides. The sugars were grafted to PLL by the same chemistry as for PLL-dextran, reaction of PLL, sugar and sodium cyanoborohydride. The presence of both PLL and sugar was shown by ^1H nmr. But quantitation of the number of sugar residues per lysine residue has not been established clearly. For PLL-glucose, the number of sugar residues appears to be about 10-20 per 100 lysine residues.

Formulations of poly IC with the PLL-ribose and PLL-glucose grafts were made. The IC-(PLL-ribose) gave a single melting transition at 78°; IC-(PLL-glucose) gave two transitions, at 66° and 77°. The 77°-78° Tm's demonstrate that a complex of poly IC with the PLL-saccharide graft exists.

Table 7. Induction of IFN in Mice by IC-(PLL-monosaccharides).

Lot	IFN titer	IFN titer of Poly ICLC	% of Poly ICLC titer
IV-161 IC-(PLL-Ribose)	52	1020	5
IV-175 IC-(PLL-Glucose)	156	1020	15

Dr. Kende's results with IC-(PLL-glucose) at 10 and 2.5 μg/mouse gave 80 and 70% survival in mice challenged with Rift Valley Fever virus, compared with 80 and 60% for poly ICLC. Thus, this formulation has substantial antiviral activity despite apparently modest IFN inducing power (Table 7).

Because the IFN titers were obtained at a standard time of 3 h after injection, pharmacokinetics may be needed in order to see the true extent of IFN induction. Also, these are initial results. The complexes of poly IC with PLL-ribose and PLL-glucose were made at a graft-to-PLL ratio of 2:1; other ratios (and other degrees of grafting) may give better results.

The data obtained to date suggest that IC-(PLL-glucose) may be as good as IC-(PLL-dextran). If so, the postulated advantages outlined in the introductory paragraph may make this inducer the preferred one.

c. β-Cyclodextrin Sulfate (βCDSO$_4$).

1. Rationale.

Carboxymethylcellulose is a polymer of high molecular weight, as are carboxymethyl dextran, the anionic amyloses and gelatin. The use of anionic cyclodextrins, small saccharides, appeared attractive for the following reasons:

1. Cyclodextrins have been described in many pharmaceutical preparations;

2. They are expected to be non-immunogenic;

3. They are small enough to be excreted, if not metabolized;

4. As small, organic compounds of definite size and structure they should be amendable to purification by common techniques of organic chemistry;

5. They should be obtainable free of microorganisms, pyrogens, etc.;

6. It may be possible to obtain more reproducible IFN inducer formulations.

7. There will be no variation of molecular weight and molecular weight distribution from batch to batch, because cyclodextrin are molecules of defined size and structure.

Cyclodextrins are cyclic oligomers of glucose, and contain 6, 7, 8 or 9 glucose residues. The 7-mer, β-cyclodextrin has been used so far in this work, because of its low cost and high solubility in water.

Sulfated cyclodextrin was prepared by the action of sulfur trioxide-trimethylamine in dimethylformamide, and was obtained as the sodium salt. A series of experiments with varied temperatures and ratios of reactants led to procedures for the synthesis of $CDSO_4$ with different degrees of substitution (DS). DS was determined from analysis for sulfur.

ii. IFN Induction.

Formulations were made with $CDSO_4$ containing 11 SO_4 groups per β-CD; one pair of lots (IV-153 and IV-190) contained twice as much $\beta CD\text{-}SO_4$ as the other pair (IV-152 and IV-191). The compositions (in mg/mL of components) and IFN titers in mice are shown in Table 8.

Table 8. ICL- $CDSO_4$; Compositions and IFN Titers.

Lot	IC	PLL	β-CD-SO_4
IV-153	2	1.5	2.5
IV-191	2	1.5	2.5
IV-152	2	1.5	5.0
IV-190	2	1.5	5.0

	Exptl. Titer	IFN titer of Poly ICLC	% of Poly ICLC
IV-153	2166	1020	212
IV-191	1481	733	202
IV-152	1000	1020	98
IV-190	755	733	103

Thus, one formulation is as effective an IFN inducer as is poly ICLC and the other is twice as effective.

The reproducibility is remarkably good, so far; but more tests will be needed. In principle one expects better reproducibility with a small molecule like $\beta CDSO_4$ than with high polymeric anions. The other inducers are made from three high polymers, which interact electrostatically (chiefly, but, no doubt, with other contributions), and may lock together in a (partially) random manner; the nature of the ternary complex may vary with slight variations in mixing procedures, molecular weights and molecular weight distributions. There may or may not be subsequent annealing (more below*). If one component at least is a small, fairly rigid molecule, one element of randomness is eliminated, and a more reproducible product may result. Additional experiments are required.

iii. Toxicity and Antiviral Action.

$\beta CD\text{-}SO_4$ itself showed no toxicity at the hig est dose tested, 250 mg/kg; LD_{50} must be substantially greater.

Table 9. LD_{50} Values for ICL-$\beta CDSO_4$ in Mice:

Inducer	LD_{50} mg/kg
Poly ICLC	11
ICL-$\beta CDSO_4$, IV-190	36
ILC-$\beta CDSO_4$, IV-191	24

Both lots of ICL-$\beta CDSO_4$ are less toxic than is poly IC. by factors of 2 and 3.5. Since IV-191 induces twice the titer of poly ICLC, its ratio of effectiveness to toxicity is correspondingly better. A pharmacokinetic study is needed.

IV-190 and IV-191 were as effective against RVFV as poly ICLC:

Table 10. Anti-RVFV Action of ICL-$\beta CDSO_4$.

Lot #	Inducer dose µg/mouse	Survivors ICL-$\beta CDSO_4$	Survivors poly ICLC
IV-190	2.5	60%	60%
IV-191	2.5	80%	60%

iv. Melting Profiles.

IV-153 and IV-191 have melting transitions, Tm, at 83°. IV-152 and IV-190 have two Tm's, at 67° and 83° in roughly equal proportions. Digestions of IV-152 by ribonuclease proceded at 60% of the rate for poly IC or for poly IC in the presence of βCDSO$_4$. (βCDSO$_4$ does not inhibit the action of RNase.) Thus, roughly half of the poly IC of IV-152 and IV-190 is uncomplexed. Since uncomplexed poly IC induces little IFN (about 10% of the titer induced by ICLC), the high titers shown above for IV-152 and IV-190 presumably arise from the that fraction of poly IC which is complexed, which means that the IFN from the latter would be equivalent (per unit weight) to the titer induced by IV-153 and IV-191, which have their poly IC completely complexed.

The presence of uncomplexed poly IC in IV-152 and IV-190, which contain twice the amount of βCDSO$_4$ as in IV-153 and IV-191, indicates that the interaction of PLL with β-CDSO$_4$ is fairly strong, resulting in the excess β-CDSO$_4$ pulling PLL out of its complex with poly IC. Attempts to prevent this by making formulations with a βCDSO$_4$ having about half the number of SO$_4$ groups per β-CD gave only insoluble complexes.

On the basis of high IFN titers, antiviral action and reduced toxicity, ICL-β-CDSO$_4$ appears to be a promising candidate.

*The question of annealing: When the three polymers are joined into a complex the initially formed complex may not be near its thermodynamically most stable structure, and there may be a gradual process of rearrangement leading to the most stable arrangement. This may be slow, and might be speeded by annealing at a temperature below the melting transition. In advance, one cannot know if this would result in superior performance, but it might result in better reproducibility.

d. Carboxymethyl-β-cyclodextrin, CMβCD.

A second approach to the use of anionic cyclodextrin is via carboxymethyl-β-cyclodextrin, CMβCD. CMβCD's of several degrees of substitution were synthesized, after working out the conditions of temperature, time, proportions of reagents and methods of isolation and purification.

1. IFN Induction.

Three formulations of poly IC, PLL and CMβCD were prepared. The induction of IFN by these agents in mice is shown in Table 11.

Table 11. IFN Induction[a] by ICL-CM-βCD.

Lot #	Composition poly IC	PLL	CM-β-CD[a]	IFN titer	% of poly ICLC titer
III-62	2	1.5	5	443	55[b]
III-254	2	1.5	5	773	143
IV-119	2	0.75	2.5	343	31

a. Degree of substitution is 4.2 carboxymethyl groups per β-CD for each lot.
b. Annual report of April 1, 1989.

The chemical difference between III-62 and III-254 on the one hand and IV-119 on the other is that the last has one-half as much PLL and CM CD.

IV-119 contained about one-half of its poly IC uncomplexed (two Tm's, at 63 and 83°), while for III-62 and III-254 all of the poly IC was complexed (Tm = 83° for III-62 and 85° for III-254).

ii. LD_{50}.

LD_{50} of III-254 was 14 mg/kg, somewhat higher than that of poly ICLC (Table 9). Lot IV-119 had LD_{50} >40 mg/kg; this lot contained half of the usual amount of PLL and CMβCD. The CM-β-cyclodextrin had an average of 0.6 carboxymethyl groups per glucose residue, or 4.2 per β-CD molecule. There are other degrees of carboxymethylation to consider, as well as α- and γ-cyclodextrin (i.e., with 6 and 8 glucose residues). In addition we are studying cyclodextrin sulfate.

Table 12. LD_{50} Values of ICL-CMβCD.

III-254	14 mg/kg
IV-119	>40 mg/kg
poly ICLC	11 mg/kg

iii. Antiviral Activity.

The action of these lots of ICL-CMβCD was tested against Rift Valley Fever Virus (III-254, IV-119) and Punta Toro virus (III-62), with the results given in Tables 13 and 14. These were as effective against these viruses as was poly ICLC. III-62 was at least as good, overall, as ICLC in all of the parameters of Table 10, % of survival, mean survival time, liver damage, enzyme assays and viral titers.

Table 13. Anti-RVFV Action of ICL-CMβCD.

Lot	Inducer dose µg/mouse	Rift Valley Fever Virus Survivors	
		ICL-CMβCD	Poly ICLC
III-254	10	9/10	9/10
	2.5	9/10	10/10
IV-119	10	6/10	5/10
	2.5	5/10	3/10

Table 14. Antiviral Action of ICL-CMβCD (Lot III-62) Against Punta Toro Virus in Mice[a,b]

Inducer dose mg/kg mouse[c]	Survivors	MLS[c]	ML VT[d] Log10	MS VT[e] Log10
III-62				
1	10/10	0.1	0.0	0.0
0.25	9/9	0.1	0.0	0.0
0.1	10/10	0.8	1.6	1.0
0.032	10/10	0.9	2.6	2.8
0.01	5/5	1.5	2.2	2.0
0.0032	5/10	2.3	2.3	3.5
Poly ICLC				
0.1	10/10	1.6	1.5	1.8
0.032	9/10	2.0	3.3	2.9
0.01	9/10	2.1	1.5	0.9
0.0032	3/9	2.7	3.4	3.2

a. Data from R.W. Sidwell, Utah State U.
b. Every other day IP Treatment.
c. Mean Liver Score, a measure of liver damage.
d. Mean Liver Virus Titer.
e. Mean Serum Virus Titer.

e. SO_4Gelatin.

Gelatin and sulfated gelatin have been investigated as replacements for CMC. Gelatin would be metabolized, and probably also sulfated gelatin. Gelatin and modified gelatins have been extensively used as plasma expanders.

SO_4gelatin work of the previous year had resulted in complexes of poly IC, PLL and SO_4gel with substantial IFN inducing ability (Annual Report, April 1, 1989). Three formulations showed moderate anti-RVFV activity in mice.

One of the earlier lots (III-111) has now been found to be digested by RNase at 33% of the rate of digestion of free poly IC. (SO_4gel does not inhibit ribonuclease.) Since the melting profile showed a single transition, at 82°, no free poly IC was present. Therefore in this ICL-SO_4gel the poly IC is significantly exposed. (Does this mean that exposed. but

complexed poly IC is able to induce IFN, or must the poly IC be dissociated from the complex to be effective?)

Lot III-198 of ICL-SO_4gel (which was an excellent IFN inducer, Annual Report, April 1, 1989), had the following effect against RVFV (data of Dr. Kende).

Table 15. Anti-RVFV Action of ICL-SO_4Gel.

Inducer dose µg/mouse	Survivors	
	III-198	Poly ICLC
10	6/10	ND
2.5	8/10	9/10

ND: not determined.

This lot was submitted for testing against Punta Toro virus (Dr. Sidwell, Utah State Univ.), with the survival results comparable to those obtained with Poly ICLC (Table 16). Other assays (enzymes and viral titers) for III-198 were similar to the averages for two trials of poly ICLC.

Table 16. Action of ICL-SO_4Gel (III-198) Against Punta Toro Virus.

Inducer dose mg/kg	Survivors		
	ICL-SO_4Gel	Poly ICLC	
		Trial 1	Trial 2
1	10/10	10/10	-
0.25	10/10	10/10	-
0.1	9/10	10/10	9/10
0.032	7/10	9/10	1/10
0.01	6/10	9/10	3/10

A new lot of ICL-SO_4gel was prepared, lot IV-261, as a repeat of lot III-199 (Annual Report of April 1, 1989). IFN induction in mice was 399 units, essentially identical to the 394 of III-199. I D_{50} of IV-261 was >40 mg/kg, the highest I D_{50} we have observed for an ICL-SO_4Gel inducer. Three earlier batches (III-106, -111, -186) had LD_{50} values of 25, 25 and 21, respectively. The high LD_{50} calls for confirmation.

Overall ICL-SO_4gel is an effective inducer, an effective antiviral agent, and is of lower toxicity than poly ICLC. In addition SO_4gel should be metabolized (to be tested by enzymic digestion).

Safety testing of ICL-SO_4Gel (IV-261) in mice resulted in a weight loss half as large as with ICLC (9.6% vs. 19.1%), but in guinea pigs IV-261 caused a larger weight loss than did ICLC (Tables 20 and 21).

It is of interest that ICL-SO_4gel is less toxic than is ICL-gel (next section). The toxicity of gelatin and SO_4-gelatin will be done to see if the difference lies in these components themselves, or arises from the complex with poly IC and PLL. If gelatin itself (and not an impurity) is toxic, and SO_4gelatin is less toxic, one might consider as a cause of this that the large number of hydrated sulfate groups conceals the surface of the gelatin preventing its interaction with some substrate. We have noted when many negative charges are added to human serum albumin it becomes non-immunogenic in mice, that is, is not recognized by the immune system.

Immunogenicity of ICL-SO_4Gel.

Two lots of ICL-SO_4gel tested earlier (Annual Report, 1989) (lots III-31A, III-31B) were antigenic in mice. But lot III-199 tested this year was not antigenic. None of the components of III-31A and III-31B was antigenic, nor did sera raised against these cross-react with SO_4gel. The earlier work was done with 100 µg of contained poly IC; the new work on III-199 was done with 10 µg of poly IC per mouse. Antigenicity of ICL-SO_4gel should be reexamined.

f. ICL-Gelatin.

In the previous year (Annual Report, April 1, 1989) an ICL-gel formulation was made with unmodified gelatin in place of CMC, lot III-210, which was an effective inducer in mice (340 units. 51% of the IFN titer of poly ICLC). Against RVFV, III-210 resulted in survival as follows (Table 17, data of Dr. Kende):

Table 17. Anti-RVFV Action of ICL-gel.

Inducer dose, µg/mouse	III-210	Poly ICLC
10	6/10	9/10
2.5	9/10	9/10

Now we have data on action against Punta Toro virus, Table 18 (data of Dr. Sidwell). III-210 was about as effective as the average of two trials for poly ICLC. Other data in these trials (enzyme assays and viral titers were comparable to the averages for the two poly ICLC trials.

A new lot, IV-243 was prepared, duplicating III-210. Lot IV-243 induced in mice 989 units of IFN, or 95% of the IFN induced by poly ICLC. Its LD_{50} was 11 mg/kg, the same as for

poly ICLC. (III-210 had not been tested for toxicity.) The potential advantage of ICL-gel is that gelatin is metabolizable. (IFN titers of these lots and of lot III-276 (below) are presented in Table 19.)

Table 18. Action of ICL-Gel (III-198) Against Punta Toro Virus.

Inducer Dose mg/kg	Survivors		
	ICL-SO_4Gel	Poly ICLC Trial 1	Trial 2
1	10/10	10/10	-
0.25	10/10	10/10	-
0.1	9/10	10/10	9/10
0.032	9/10	9/10	1/10
0.01	6/10	9/10	3/10

ICL-Gel previously prepared had been made with a gelatin of isoelectric point (IP) of pH 9, an acid-process gelatin. At physiological pH this will have a slightly positive net charge. We have now prepared ICL-gel with a base-process gelatin, with isoelectric point of pH 5, which has a negative net charge at physiologic pH. This lot, III-276, gave an IFN titer of 856 units or 56% of that of poly ICLC, a value comparable to those of ICL-Gel formulations made with acid process gelatin (which runs from about 50 to 95%). Thus, both major types of gelatin give effective inducers. A choice between them would depend on other properties, such as toxicity, immunogenicity, antiviral action, etc.

Although the two types of gelatin have different net charges, they contain both positive and negative charges but in different proportions. This may result in differences in the organization of the complexes with poly IC and PLL, which could affect the rate of digestion of the poly IC and the kinetics of IFN induction. Twice as much poly(L-lysine) is required to complex the poly IC when IP 5 gelatin was used as when IP 9 gelatin was used. The net negative charge on IP 5 gelatin (resulting from a greater number of negative carboxylate groups) would probably bind cationic poly(L-lysine) more strongly than does IP 9 gelatin (net positive charge), thereby competing more effectively against poly IC for binding to poly(L-lysine) and requiring more poly(L-lysine) to form the ternary complex.

Melting profiles were done on III-276, III-210 and IV-243. III-276 gave two melting steps, 63° and 79.5°, in the proportion of 40% to 60%, respectively. The 63° Tm is probably that of free poly IC. III-276 was less toxic than poly ICLC (Table 9).

III-210 and IV-243 had Tm values of 80° and 82°, respectively; all poly IC is complexed.

Overall, ICL-gelatin is an effective inducer, and effective antiviral agent. LD_{50} must be resolved as to the source of the difference between III-243 and III-276. The advantage of ICL-gel is that all components are metabolizable.

Table 19. IFN Induction and LD_{50} by ICL-Gelatin in Mice.

	IFN Induction			LD_{50}
	Experimental	ICLC Standard	% of ICLC	
ICL-Gel (IP = 5) III-276 2:1.5:5[a]	856	1535	56	21 mg/kg
ICL-Gel (IP = 9) III-210 2:0.75:6[a]	340	670	51	
ILC-Gel (IP = 9) IV-243 2:0.75:6[a]	989	1039	95	11 mg/kg

[a]. Concentrations of IC, PLL and Gelatin in mg/mL.

g. Amylose Sulfate ($AmSO_4$).

We have begun studying sulfated amylose and amylopectin. Good results had been obtained with carboxymethyl amylose (CMA). The rationale was that sulfatases may hydrolyze the sulfate esters, restoring the amylose, which could then be metabolized. We have prepared $AmSO_4$ of several degrees of sulfation by reaction of amylose with sulfur trioxide-trimethylamine complex in dimethylformamide. Reaction conditions (time, temperature and ratios of reactants) to obtain different degrees of substitution were worked out. (Amylopectin sulfate has also been prepared, but no biological data have yet been obtained.)

Formulations were made with poly IC, PLL and $AmSO_4$ at DS = 0.4, 0.7 and 1.4 (sulfate per glucose residue), and melting profiles were obtained (Table 20).

Table 20. Melting Transitions of ICL-Amylose-SO_4.

Lot No.	Composition, IC:PLL:Amylose-SO_4 (by weight)	DS of Amylose-SO_4	Tm, °C
IV-212	2:0.75:2.5	0.4	84
IV-211	2:0.75:2.5	0.7	68
IV-210	2:0.75:2.5	1.4	64
IV-212A	2:1.5:5	0.4	84.5
IV-211A	2:1.5:5	0.4	64

The Tm data show that complexes were formed with amylose-SO_4 of DS 0.4 at both proportions of PLL and amylose-SO_4, but no complex was observed at the higher DS values. (The Tm of 68° for IV-211, being 4° higher than for poly IC alone, may indicate a complex. This has not been established, because ribonuclease digestion cannot be used, because $AmSO_4$ is an inhibitor of RNase.) Apparently at high DS, the negative charge on amylose-SO_4 pulls PLL out of interaction with poly IC, leaving bare poly IC.

The first ICL-$AmSO_4$ (IV-212) to be tested in vivo induced in mice an IFN titer of 183 units or 18% of that of poly ICLC. This is in the same range as the titers induced by IC-(PLL-dextran). The time course of IFN induction should be done, in order to see if this might be an effective inducer. Also, lower degrees of substitution may be useful. Amylopectin sulfate has been synthesized, but not yet tested in IFN inducer formulations.

h. Carboxymethyl Amylose (CMA).

ICL-CMamylose has given good results as IFN inducer and antiviral agent. Therefore, we have devoted more effort to this candidate formulation. The original lot of amylose was exhausted, so a new preparation of CMamylose was made, using a new batch of amylose. This gave ICL-CMamylose formulations of low IFN inducing ability (III-246, III-248, and III-255, Table 21). That this was not a defect in our technique of formulation was shown by making a new batch of ICL-CMamylose equivalent to III-107 (reported earlier) from some remaining original CMamylose; this batch, III-258, gave 247% as much IFN as did standard ICLC. A third batch of amylose was carboxymethylated. The ICL-CMamylose formulations from this gave good IFN titers, 989 units, and 534 units, or 64% and 35% of standard ICLC for two formulations differing in the PLL content.

There was still a difference from ICL-CMamylose batches made with the original first batch of amylose, namely, that twice as much PLL was needed with batch-3 CM-amylose to complex all of the IC as with batch-1. That is, III-278 had two Tm values, 63° and 81°, while III-281 had only an 81° Tm (Table 22).

The difficulty with the second batch of amylose suggests that one cannot depend on amylose batches to be reproducible. At present we do not know what properties of amylose are critical. We must also bear in mind that the low IFN titers of formulations from the second batch of amylose may have been only a change in kinetics, resulting from a subtle variation in the amylose (molecular weight, molecular weight distribution, etc).

Comparison of III-278 and III-281 is instructive. III-278 had one-half as much PLL as did III-281. III-278 had two Tms, at 62° and 81° (in the ratio of 40:60 for A), and III-281 had only one Tm, at 81° (Table 22). III-281 had nearly twice the IFN titer of III-278. In this case we can think that the 62° Tm of III-278 is that of IC alone, since it vanished with more PLL. Also, since III-281 had almost twice as much of the 81° complex and twice the IFN titer, it appears that the IFN titer may be proportional to the concentration of complexed IC..

Table 21. IFN Induction in Mice by ICL-CMamylose.

Lot and Composition[a]	Amylose Lot	IFN Induction		
		Exptl. Formulation	ICLC Standard	% of ICLC Standard
III-246 2:0.75:2.5	2	46	799	6
III-248 2:0.75:2.5	2	5	799	1
III-255 2:1.5:2.5	2	127	1535	8
III-258[b] 2:0.75:2.5	1	1329	539	247
III-278 2:0.75:2.5	3	534	1535	35
III-281 2:1.5:2.5	3	989	1535	64

[a] Composition in mg/mL of the components in the order: IC, PLL, CMamylose.

[b] Equivalent to III-107 (Annual Report, April 1, 1989, Table 3).

ii. Poly(L-arginine).

With poly IC, PLA and CMC an insoluble complex formed, when these components were mixed in the same ratio as for poly ICLC. With the proportion of PLA reduced by one-half (poly IC and CMC held at the standard concentration), a clear solution (lot IV-111) was obtained, but the melting transition was low, 67°, and no significant titer of IFN was induced.

The 67° Tm for poly IC-PLA-CMC, compared with 63-64° for poly IC suggests that a complex may have been formed, and this is supported by the formation of a precipitate in the case where more PLA was used. The nearly zero IFN titer for IV-III, compared with about 100 for free poly IC, may mean that IC, PLA and CMC are bound in a complex of low activity, or the pharmacokinetics are different.

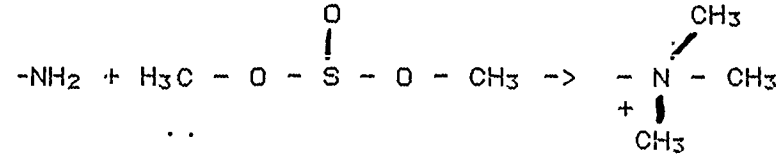
The relatively low Tm of 67°, compared to 75°-95° for most complexes of poly IC with other components, does not necessarily mean that binding of PLA to poly IC is weak; it can mean that binding to denatured poly IC is relatively strong, since the Tm of a complex of a polynucleotide depends on the relative strength of binding of the ligand to helical and to denatured polynucleotide.

iii. Modified Graft Polymers.

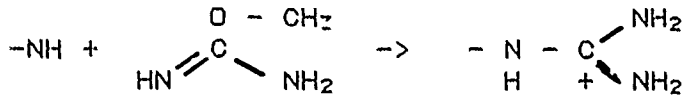
Formulations of poly IC with methylated and guanidinated PLL-dextran graft polymers had melting transitions of 63-64°, the same as for poly IC alone. Apparently, no complex was formed. The result for guanidinated PLL-dextran is unexpected, as strengthened binding is expected.

These results suggest that ammonium groups of PLL and of PLL-dextran have near-optimum binding properties in poly ICLC. It is possible that binding might be fine-tuned by partial chemical modification of PLL, or by the use of copolymers of lysine with other amino acids. With anionic replacements for CMC, PLL might not be optimum.

Methylation was carried out by treating PLL or PLL-dextran with dimethyl sulfate in water at pH 9-10, followed by thorough dialysis. The reaction is:



The methylated amine is a quaternary ammonium ion, positively charged at all pH values. Guanidination was carried out with O-methyl-isourea sulfate in water at pH 9-10:



The guanidium group is positively charged.

j. Miscellaneous Formulations.

i. Several other polyanions were tested as replacements for CMC. The rationale in each case was ease of digestion or excretion, and biological compatibility. The polyanions were:

Poly(guanylate), PG

Poly(uridylate), PU

Poly(galacturonic acid), PGal

Two formulations with PG, using different ratios of PLL and PG to poly IC gave 19 and 13% of IFN titer of poly ICLC. The poly IC is probably not complexed, as the Tm's were about 65°.

A formulation of poly IC, PLL and PU (composition 2:1.5:5) gave 14% of the IFN titer of poly ICLC. The melting transition of ICL·PU was 68°, 3-4° higher than that of poly IC.

A formulation of poly IC, PLL and PGal gave 4% of the IFN titer of poly ICLC. The melting profile showed two Tm values, 83° and 94°; both are for complexed poly IC.

ii. We also replaced PLL by a random copolymer of lysine and alanine. This "dilutes" the positive charges on PLL and increases hydrophobicity. The copolymer had the composition 67% Lysine 33% alanine, or a 2:1 ratio, and is designated L_2A. A formulation (IV-78) containing IC, L_2A and CMC in the proportions 1:1.5:2 by weight induced in mice an IFN titer of 130 units, or 14% of that of poly ICLC. Thus, replacing one-third of the lysine residues by alanine has reduced the IFN titer (or changed the time course of its induction). Tm for IV-78 was 75°, demonstrating the existence of a complex.

iii. Heat-annealed poly ICLC.

Elsewhere in this report we have raised the question of annealing of an inducer. We have tested this idea in an extensive form by heating poly ICLC above Tm, then cooling during one hour to room temperature. This would allow the three polymeric components to interact more slowly than by direct mixing.

Table 22. Melting Temperatures of ICL-CMamylose.

Lot and Composition[a]	Tm 1 °C	% of Total ΔA	Tm 2 °C	% of Total ΔA
III-246 2:0.75:2.5	61	55	80	45
III-248 2:0.75:2.5	63	25	80	75
III-255 2:1.5:2.5	62	80	82	20
III-258 2:0.75:2.5	-	-	79	100
III-278 2:0.75:2.5	62	40	81	60
III-281 2:1.5:2.5	-	-	81	100

a. Composition in mg/mL of the components in the order: IC, PLL, CMamylose.

Table 23. LD_{50} of ICL-CMamylose and Other Substances.

Substance	LD_{50}
Poly ICLC Standard	11 mg/Kg[a]
ICL-CMA III-281	25 mg/Kg
Poly IC	33 mg/Kg[b]
PLL	>30 mg/Kg
CMC	>75 mg/Kg

a. Average of four measurements: 13, 11 and 9 mg/Kg.
b. Average of three measurements: 26, 37 and 35 mg/Kg.

ICL-CMamylose complex III-281 gave an IFN titer of 64% of that of the ICLC standard (989 units vs. 1535 units, Table 21). LD_{50} of this preparation and those for the poly IC and CMC batches we are using are shown in Table 23.

Thus, ICL-CMA III-281 has more than twice the LD_{50} of ICLC. It is notable that poly IC alone is less toxic than is ICLC or ICL-CMA. Also, CMC is less toxic. However, the combination of

ingredients tends to be more toxic, especially for poly ICLC. Lot III-107 of ICL-CMA had been shown to be effective against RVFV (Annual Report, April 1, 1989, Table 1). Against Punta Toro virus III-107 was effective at doses of 10 and 2.5 mg/kg, but not at 0.1 mg/kg (Table 24). (There were no data between 2.5 and 0.1 mg/kg.)

Table 24. Anti-Punta Toro Action of ICL-CMA (III-107).

Inducer dose mg/kg	Survivors	
	III-107	poly ICLC
10	9/10	10/10
2.5	10/10	10/10
0.1	1/10	19/20

i. Methylated and Guanidinated PLL and PLL-Dextran Graft Polymers.

Is PLL the optimum cationic polypeptide? The PLL used in formulations with poly IC and polyanions and in making graft polymers with dextran, has a certain degree of binding strength toward poly IC and toward CMC or a replacement polyanion. This binding strength might not be optimum for our purpose. Therefore, we investigated chemically modified PLLs which would have weaker or stronger binding to poly IC or to a polyanion.

Methylation of the amino groups weakens binding to polynucleotides, as measured by the ability of salt to dissociate the cationic polypeptide from polynucleotide (5). Guanidinated amino groups strengthen binding . Therefore we methylated PLL to poly(N^{e},N^{e},N^{e}-trimethyl-L-lysine) (PTMLL) (5), and we methylated and guanidinated a PLL-dextran graft. In place of guanidinated PLL we used poly(L-arginine), PLA.

1. Methylated Poly(L-lysine).

A mixture of poly IC, PTMLL and CMC showed a melting transition similar to that of poly IC. Apparently the poly IC was free, not bound to PTMLL. The formulation did not induce any significant titer of IFN, and gave, in fact, values of about 6 units/mL, essentially nil. The binding of PTMLL to poly IC may be weakened more than its binding to CMC, so that CMC outcompetes the poly IC. In the absence of CMC PTMLL does bind to polynucleotides including poly IC, but more weakly than does PLL. (5).

The result was a total loss of IFN induction and antiviral action against Punta Toro virus. Heating above Tm is too drastic. We hypothesize that the separated strands of poly I and poly C may have interacted with PLL and CMC before reforming the double helix of poly IC. More gentle annealing below Tm (i.e., below the temperature at which the poly IC double helix is dissociated) may be better.

k. Safety testing.

Several types of formulations were tested for safety in mice and guinea pigs: ICL-SO_4Gel, ICL-βCDSO_4, ICL-CMA, IC-(PLL-dextran) and poly ICLC, with the results shown in Tables 25 and 26. The results are discussed in the sections on individual IFN inducers. Overall, all of our experimental formulations resulted in smaller weight losses in mice than did poly ICLC. In guinea pigs, ICL-SO_4gel, one lot of IC-(PLL-dextran) and a lot of ICL-CMA caused larger losses than did poly ICLC at 48 hours, but all recovered and showed weight gains as large or larger than did controls which received only saline. Two lots of ICL-βCDSO_4 and one of IC-(PLL-dextran) resulted in weight gains at 48 hours.

Table 25. Safety Testing in Mice[a]

Compound	Wt. loss or gain, % at 48 hr
Saline	+3.2%
ICLC (BG-IV-241)	-19.1%
ICLSO_4Gel (BG-IV-261)	-9.6%
ICL-βCDSO_4 (BG-IV-190)	-11.8%
ICL-βCDSO_4 (BG-IV-191)	-15.5%
ICLCMamylose (BG-III-107)	-15.0%
IC(PLL-Dextran) (BG-IV-258)	-9.1%
IC(PLL-Dextran) (BG-IV-259)	-8.5%

[a] 4 mice per group. Dose: 8 mg/kg (160 µg/mouse), as poly IC.

Table 26. Safety Testing in Guinea Pigs[a]

Compound	Wt. loss or gain, % at 48 hr
Saline	+3.9%
ICLC (BG-IV-241)	-4.5%
ICLSO_4Gel (BG-IV-261)	-13.1%
ICL-βCDSO_4 (BG-IV-190)	+3.8%
ICL-βCDSO_4 (BG-IV-191)	+3.7%
ICLCMamylose (BG-III-107)	-8.9%
IC(PLL-Dextran) (BG-IV-258)	-9.0%
IC(PLL-Dextran) (BG-IV-259)	+3.6%

[a] Two guinea pigs per group. Dose, 8 mg/kg, vs poly IC.

6. Conclusions.

Our research has resulted in the demonstration that effective IFN inducers can be formulated without using carboxymethylcellulose. Our new formulations have shown effective IFN induction and antiviral activity, some of them against two viruses, Rift Valley Fever and Punta Toro. Most of them are less toxic than is poly ICLC.

An important finding was that an IC-(PLL-dextran) which was a moderate inducer of IFN in mice, under our standard protocol (blood tested for IFN three hours after injection) was as effective an inducer as poly ICLC in a monkey, and gave a peak titer earlier than did poly ICLC.

Many formu ations were tested for antigenicity; none was found to be antigenic by the precipitation-in-gel method, except two lots of ICL-SO_4gel. A more recent lot of ICL-SO_4gel was not antigenic.

The effective interferon inducers are:

IC-(PLL-dextran)

ICL-CMamylose

ICL-CM-β-cyclodextrin

ICL-β-cyclodextrin sulfate

ICL-CMdextran

ICL-gelatin

ICL-SO_4gelatin

ICL-β$CDSO_4$ (2 lots tested) had an LD_{50} 2.7 times as high as poly ICLC (Table 27), good IFN induction (Table 28) and good antiviral action (Table 29). The β-$CDSO_4$ itself was non-toxic).

ICL-SO_4 gelatin (ICL-SO_4gel) had an average LD_{50} 2.5 times that of poly ICLC (Table 27), good IFN induction (Table 28), but was somewhat less effective than poly ICLC against RVFV. However, against Punta Toro virus it was at least as effective as poly ICLC (Table 16). The probability that SO_4 gelatin is metabolized to amino acids is an attractive feature of this inducer.

ICL-CMA (ICL-CMamylose) had an LD_{50} of 2.3 times that of poly ICLC (Table 27), and was a good IFN inducer (Table 28) and antiviral agent (Table 29). CMamylose has been reported to be safe at doses more than 1000 times greater than ours (as a blood volume expander, ref. 4). The chief drawback is the unsuitability of some batches of amylose; but this can be checked, and good batches can be used.

The inducer types effective against viruses were:

Inducer Type	Rift Valley Fever	Punta Toro
ICL-CMamylose	+	+
ICL-gelatin	+	+
ICL-SO_4gelatin	+	+
ICL-CM-β-cyclodextrin	NT*	+
ICL-CMdextran	−	+
ICL-(PLL-glucose)	+	NT*

*Not tested.

Table 27. Summary of LD_{50} Values.

Inducer		LD_{50} (mg/kg)	(average
Poly ICLC		11	(average of 5 lots)[a]
IC-PLL-Dext (See Table 2 for individual LD_{50}'s)			
6 + 10[b]		31	(average of 2 lots)
6 + 70		27	(1 lot)
22 + 10		>35	(average of 5 lots)
63 + 10		>44	(average of 2 lots)
63 + 40		37	(average of 2 lots)
			overall average: >35
ICL-βCDSO$_4$	IV-190	36	average 30
	IV-191	24	
ICL-CMβCD	III-254	14	
	IV-119	>40	
ICL-SO$_4$Gel	IV-261	>40	
	III-106	21	average 28
	III-111	25	
	III-186	25	
ICLGel	IV-243	11	
ICL-CMA	III-281	25	average 25
	III-107	25	

a. Individual LD_{50}'s: 9,10,11,11,10.
b. The two numbers refer to the molecular weights of the PLL and Dextran, respectively, in thousands. The LD_{50} values of the 12 lots are displayed in Table 2.

The LD_{50} values of the several classes of IFN inducers are summarized in Table 27. The most extensively studied was IC-(PLL-dextran), 12 lots of which were tested and gave an average LD_{50} greater than 35 mg/kg, or more than three times the LD_{50} of poly ICLC. Although its average IFN titer (at our standard time of 3 hours after injection into mice) was 17% of that of poly ICLC (Table 28), IC-(PLL-dextran) was an effective anti-viral

agent (Table 29). Either 17% is adequate, or a higher titer is achieved at some time after the 3-hour test. The initial monkey result (Table 3) gave a high titer for a batch of IC-(PLL-dextran).

Table 28. Summary of IFN Induction (as % of the Titer Induced by Poly-ICLC).

IC-(PLL-Dextran)	17%	(ave. of 24 lots)
IC-PLL-glucose)	15%	(one lot)
ICL-βCDSO$_4$	154%	(ave. of 4 lots)
ICL-CMβCD	76%	(ave. of 3 lots)
ICL-SO$_4$gelatin	63%	(ave. of 12 lots)[a]
ICL-gelatin	67%	(ave. of 3 lots)
ICL-CMamylose	115%	(ave. of 3 lots)[b]

a. Annual Report of April 1, 1989).
b. Does not include 3 lots made with Amylose Lot #2. See Table 16 and relevant text, pp 34-35.

Table 29. Summary of Anti-RVFV Action in Mice.

	% Surviving Mice	
	Experimental Lot	Poly ICLC
IC-(PLL-Dextran)	93 (13 lots)	78
ICL-βCDSO$_4$	70	60
ICL-βCMCD	73	68
ICL-gelSO$_4$	70	90
ICL-gel	75	90
ICL-CMamylose[a]	80	80

a. Report of April 1, 1989.

Overall, this is a promising antiviral candidate.

The next major task is to select from these candidates the best one or two for eventual clinical trials. To this end we shall address the following points:

1. Reproducibility and stability of formulations, especially on larger scale preparations.

2. Time course of IFN induction.

3. Optimum proportions and molecular weights of components. (Some of this has been done.)

4. Further testing of LD_{50}, to obtain reliable statistics.

5. Safety testing, pyrogenicity testing.

6. Assay of enzymes associated with IFN induction.

7. Test digestion of formulations and components by mammalian enzymes to verify that they are likely to be metabolized.

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8. Glossary.

A:	Absorbance (spectrophotometer)
$AmSO_4$:	Sulfated amylose
CM:	Carboxymethyl
CMC:	Carboxymethylcellulose
CMβCD:	Carboxymethyl-β-cyclodextrin
CMamylose:	Carboxymethyl amylose
CMA:	Carboxymethyl amylose
$\beta CDSO_4$:	β-Cyclodextrin sulfate
CMdextran:	Carboxymethyl dextran
Gel:	Gelatin
ICL:	Complex of IC with PLL
ICLC:	Complex of IC with PLL and CMC
ICL-CMamylose:	Complex of IC, PLL and CMamylose
ICL-CMA:	Complex of IC, PLL and CMamylose
ICL-CMdextran:	Complex of IC, PLL and CMdextran
IFN:	Interferon
kDa:	Molecular weight in thousands
PLL:	Poly(L-lysine)
Poly IC; IC:	PolyI·PolyC
PTMLL	Poly(Trimethyl-L-lysine)
RNase:	Ribonuclease
RVFV	Rift Valley Fever virus
SO_4Gel	Sulfated gelatin
Tm:	Melting, or transition, temperature

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